The relationship between cyclobutane pyrimidine dimer photolyase activity and disease resistance to the rice blast fungus Magnaporthe oryzae in African rice

氏名（本籍地）	ギデオン　サディキエール　ムバンド GIDEON SADIKIEL MMBANDO
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論文題目	The relationship between cyclobutane pyrimidine dimer photolyase activity and disease resistance to the rice blast fungus Magnaporthe oryzae in African rice（アフリカイネにおけるシクロブタン型ピリミジン二量体光回復酵素活性とイネいもち病菌 Magnaporthe oryzae に対する病害抵抗性との関係）
博士論文審査委員	（主査）　日出間　純 東谷　篤志 佐藤　修正 植田　美那子

論文内容の要旨

Ultraviolet B (UVB, 280–320 nm) sensitivity varied widely among Asian (*Oryza sativa* L.), and the activity of cyclobutane pyrimidine dimer (CPD) photolyase, which repairs UVB-induced CPDs, determine the UVB sensitivity [1]. The UV-resistant rice has significantly increased CPD photorepair ability compared to UV-sensitive rice due to variation of CPD photolyase activity resulted from natural occurring polymorphisms within CPD photolyase gene [2–4], and thus the UVB sensitivity is highly correlated to CPD photolyase activity. In fact, UVB resistant CPD photolyase overexpress transgenic plants have been generated using Asian rice cultivars, with significant improved in UVB resistant than wild type plants [1,5], suggesting that CPD photolyase is crucial factors for determining the UVB sensitivity. UVB radiation can also induce defensive mechanisms and reduce oxidative damage [6], boost plant defense system against pathogenic fungi [7], such as *H. parasitica* [8] and *Botrytis cinerera* [9].

African rice (*Oryza glaberrima* Steud. and *Oryza barthii* A. Chev.) is a different species, that differs from *O. sativa* in many qualitative and quantitative traits such as differences in ligule shape, panicle branching, tiller number formation and growing phenotype; and it has independently domesticated in West Africa by possess traits for increased tolerance to biotic and abiotic stresses such as drought, soil acidity, iron and aluminium toxicity [10,11]. Thus, one might expect that rice cultivated in tropical areas of Africa must have developed some UVB resistant mechanisms. However, the information about UVB sensitivity of African rice, generation of UVB resistant transgenic rice plant is largely absent, possibly due to challenge in transformation and regeneration of African local cultivar. Although African rice have shown to be resistant to several biotic stresses, the information about the interaction between UVB sensitivity and resistance to the rice blast fungus *Magnaporthe oryzae* have remained unexplored. Thus, the aim of this study was to understand the UVB sensitivity of African rice, generate of UVB resistant transgenic African rice, and examine the relationship between UVB sensitivity and resistance to the rice blast fungus *M. oryzae* in African rice.

Chapter 1 The UVB sensitivity is highly correlated with CPD photolyase activity [3,4]. Although it grown in tropical environment with high UVB, the UVB sensitivity and CPD photolyase activity of African rice has remained largely unknown. In chapter I, I investigated the UVB sensitivity and CPD photolyase activity of

15 African rice cultivars. To achieve this purpose, 15 Africa cultivated rice including *O. glaberrima*, *O. barthii* and tropical *O. sativa* were grown under visible radiation with or without 1.2 W m^{-2} of supplementary UVB in growth chamber. Unexpectedly, most of African rice cultivars were more sensitive to UVB radiation (UVB super-hypersensitive) than even the hypersensitive Asian rice Surjamkhi [2] (Fig. 1A, B). The UVB super-hypersensitive cultivars have newly identified polymorphism of S^{78}-R^{126}-A^{283}-Q^{296} in comparison to P^{78}-Q^{126}-G^{283}-Q^{296} of UVB resistant Asian rice Sasanishiki (Fig. 1C). The activity of purified CPD photolyase of selected African rice (Fig. 1D, E) and total CPD photolyase activity (Fig. 1F), was significant lower in UVB super-hypersensitive and Surjamkhi compared to Sasanishiki and Norin 1[12]. These results suggested that similar to Q to H^{296} on Surjamkhi[2], polymorphisms of P to S^{78} and G to A^{283} largely reduces the CPD photorepair activity of UVB super-hypersensitive cultivars, and enhancing CPD photolyase activity can improve the UVB resistance of African rice.

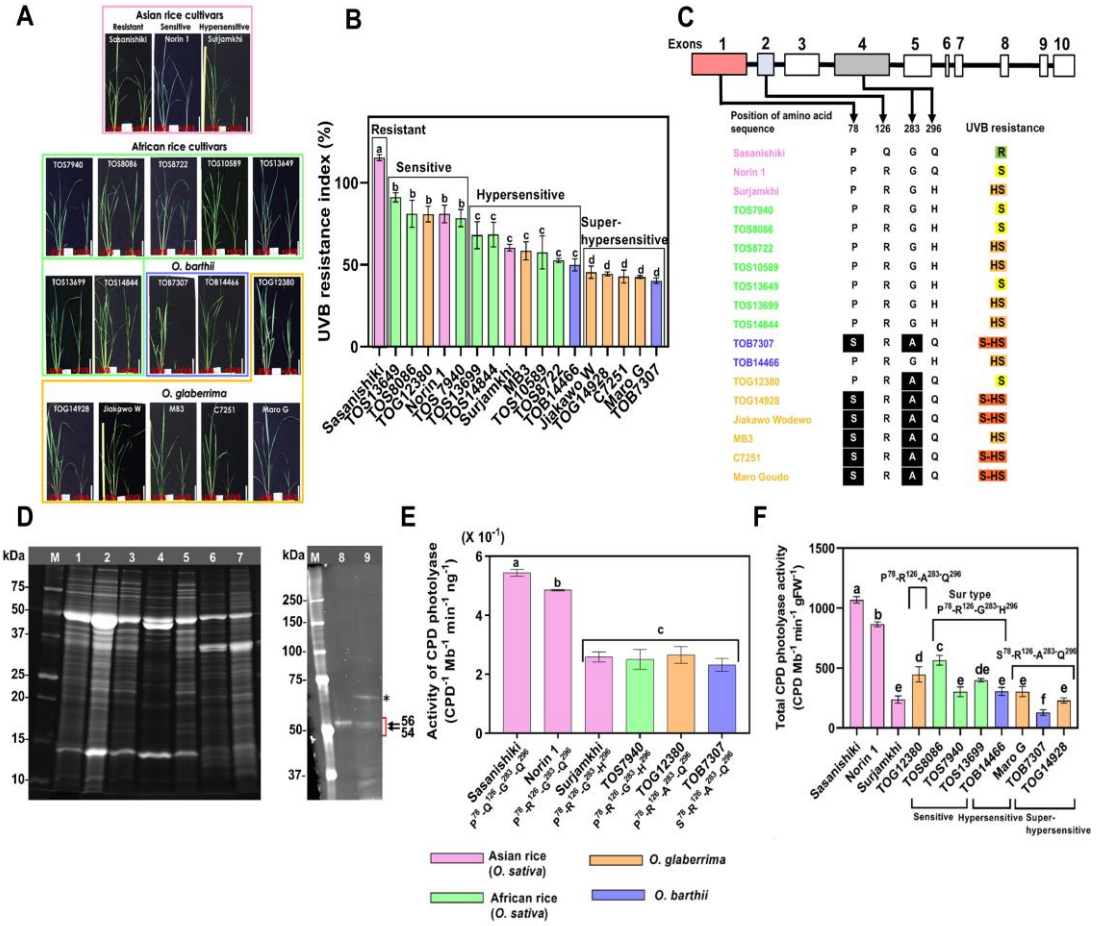

Figure 1. UVB sensitivity, CPD photolyase genotype and activity of African rice in comparison to Asian rice cultivars (A) Rice plant of three species (*Oryza sativa, O. barthii* and *O. glaberrima*) were grown in a growth cabinet for 21 days with (+UVB) or without (-UVB) UVB radiation. Jiakawo W, Jiakawo Wodewo; Maro G, Maro Goudo. UVB resistance differed widely among African rice cultivars. Bars = 5 cm. (B) UVB resistance index was calculated by summing the value of the ratio of +UVB to -UVB

of tiller number and above-ground fresh weight × 100, i.e., (+UVB)/(-UVB) × 100. (C) CPD photolyase genotypes found in African rice, P^{78}-R^{126}-G^{283}-H^{296}, P^{78}-R^{126}-A^{283}-Q^{296} and S^{78}-R^{126}-A^{283}-Q^{296}. Amino acid residues highlighted in black (S^{78} and A^{283}) were mostly found in African rice cultivars that are UVB-super-hypersensitive compared to Sasanishiki. UVB resistance: R = resistance, S = sensitive, HS = hypersensitive and S-HS = super-hypersensitive. (D) Purified native CPD photolyase protein from whole plants African rice cultivar TOS7940. Electrophoresis was performed in a 12.5% or 7.5% SDS-polyacrylamide gel, which was stained with SYPRO Ruby stain. Arrows indicate phosphorylated (56 kDa) and unphosphorylated (54 kDa) CPD photolyase. The asterisk indicates that BSA was detected in our final purification fraction (E) CPD photorepair activity of purified native CPD photolyase. (F) Total CPD photolyase activity, calculated from the CPD photolyase protein content [(pg of CPD photolyase)/(μg of soluble protein)], total soluble protein content (μg/gFW) and activity of CPD photolyase (CPD/Mb/min/ng). Each sample was harvested form 9 plants, median and SD were calculated from three biological independent experiment, each performed in triplicate in B, E and F; different letters indicate significant differences determined by the Tukey-Kramer test ($P < 0.05$).

Chapter 2 Overexpress CPD photolyase in transgenic plants have shown to improve the UVB resistant of Asian rice cultivars[1,5]. However, at current moment, to my best knowledge, there are no any reports that has generated the UVB tolerant transgenic African rice using TOG12380 (*O. glaberrima*) as parental line (PL) plant, possibly due to the difficulties in the transformation and regeneration of African local cultivars. Therefore, in chapter 2, I investigated about the creation of UVB resistance transgenic African rice cultivar. To achieve this purpose, the CPD photolyase gene of UVB resistant Asian rice Sasanishiki was ubiquitously expressed by the CaMV 35S promoter using UVB sensitive TOG12380 (*O. glaberrima*) as PL plant (Fig. 2A). The transgenic overexpress African rice (TOG12380-OxPHR) have significant improved in UVB resistance (Fig. 2B), with higher transcripts (Fig. 2C) and activity (Fig. 2D) of CPD photolyase than PL plants. These results strongly suggest that despite of species barrier, UVB-induced CPDs is still the main causes for UVB-induced growth hindrance in higher plants grown under supplementary UVB radiation stress, and CPD photolyase is proper bioengineering tools for developing the UVB resistant African rice.

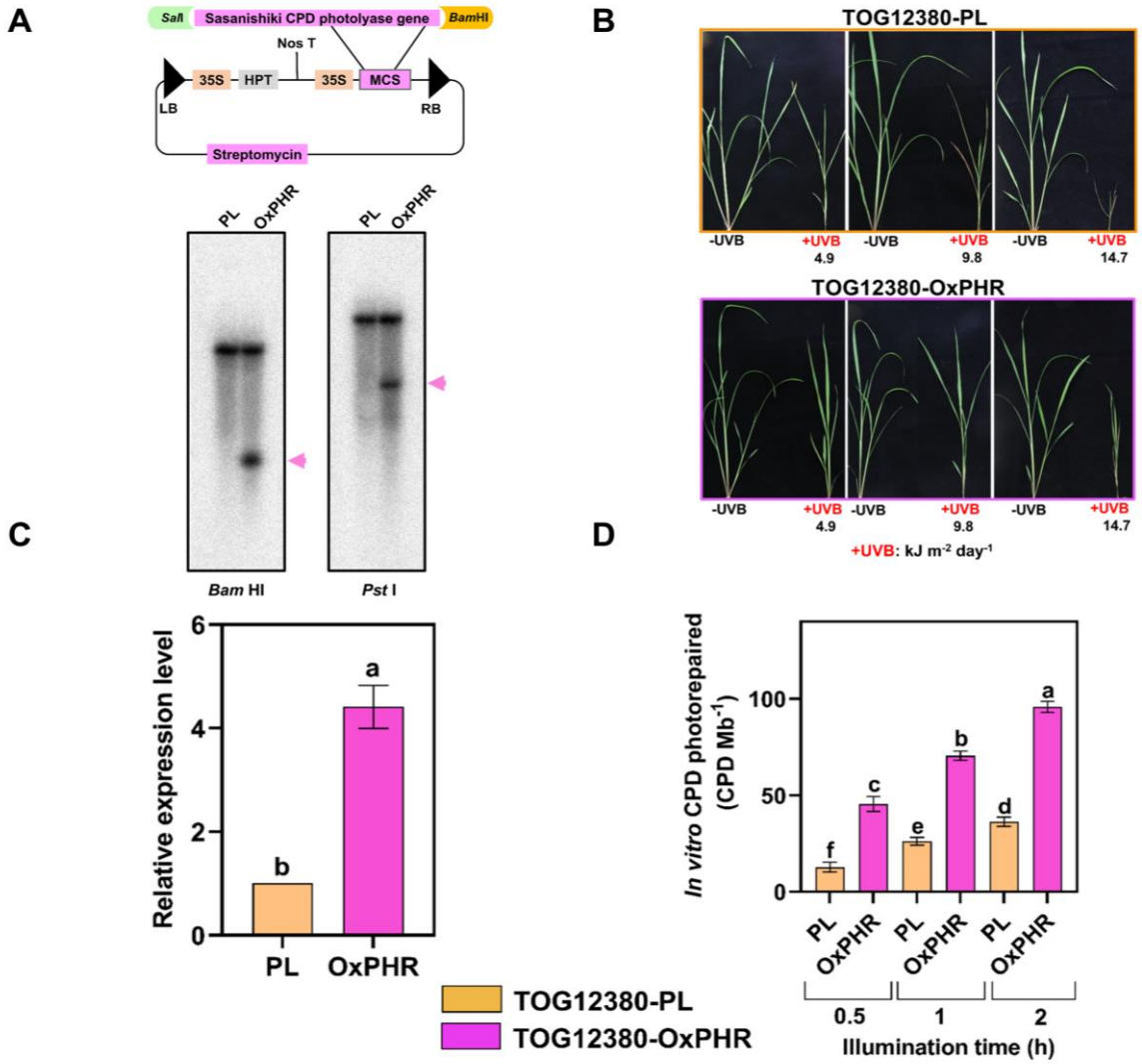

Figure 2. CPD photolyase copy number, transcript level and activity in transgenic African rice overexpressing CPD photolyase (TOG1230-OxPHR). (A) Transform construct pPZP2Ha3 that was used to transform rice with cDNA encoding the CPD photolyase of the Sasanishiki cultivar. The cDNA was subcloned into a multi-cloning site (MCS) of the binary vector pPZP2Ha3 in the sense orientation. *Bam*HI and *Pst*I are the restriction sites used for Southern blot. Southern blot analysis of the rice CPD photolyase gene in parental line (TOG12380-PL) and overexpressing transgenic plants (TOG12380-OxPHR). The magenta arrows head indicate the CPD photolyase gene bands. (B) Photographs of plants grown for 21 days under visible radiation supplemented with [(+UVB); (4.9, 9.8 and 14.7 kJ m^{-2} day^{-1})] or without (−UVB) UVB radiation. (C) Ratios of CPD photolyase transcripts (measured by quantitative real-time RT-PCR analysis) of Ox-PHR transgenic plants relative to that of the PL plant. Actin was used as an internal control. The level of PL was set to be 1. (D) *In vitro* CPD photorepair activity in the crude soluble protein of TOG12380-OxPHR and TOG12380-PL. Values are means ± SD. n = 4 replicates; different letters indicate significant differences determined by the Tukey-Kramer test ($P < 0.05$).

Chapter 3 UVB radiation can induce defensive mechanisms and reduce oxidative damage [6], boost plant defense system against pathogenic fungi [7], such as *H. parasitica* [8] and *Botrytis cinerera* [9], both in Arabidopsis. The fact that UVB-hypersensitive (Surjamkhi) and super-hypersensitive (TOB7307) cultivars have been domesticated and are still cultivated today in tropical area of Bengal [13,14] and Africa [12], respectively, prompt me to ask whether such a high UVB sensitivity may be beneficial for surviving other stresses in tropical areas as pathogens. However, the relationship between CPD photolyase activity and resistance mechanisms to pathogens is poor understood. Thus, in chapters 3, I investigated the relationship between CPD photolyase activity and resistance to the rice blast fungus *M. oryzae.* To achieve this purpose, *M. oryzae* basal resistance was analysed by punch and leaf sheath assay on transgenic plants with different CPD photolyase activity (S-C line); Sasanishiki CDP photolyase overexpressed and (AS line); antisense line with high and low CPD photolyase activity [1], respectively, and TOG12380-OxPHR and TOG12380-WT, with high and low CPD photolyase activity, respectively. I discovered that 12 hours of 0.4 W m^{-2} UVB pretreatment, reduces secondary infection hyphae of AS line compared to S-C and Sasanishiki (Fig. 3A, B). Interestingly, UVB pretreatment enhances susceptibility to rice blast on the UVB resistant transgenic lines S-C (Fig. 3C) and TOG12380-OxPHR (Fig. 3D), with high CPD photolyase activity. Moreover, UVB pretreatment did not activates expression of OgJiPR10, OgWRKY45 and CAD 6 PR genes of TOG12380-OxPHR plants (Fig. 3E). Thus, similar to UVC-induced DNA damage that activate immune response prior to *H. parasitica* molecules recognition by plant [8], these results suggested that the high UVB-induced DNA damage in AS line and TOG12380-WT, due to low CPD photolyase activity, activates expression of PR genes and enhances basal resistance of *M. oryzae* prior to infection occurrence (Fig. 3C, D and E).

Conclusion In this study, I showed that African rice cultivars are highly sensitive to UVB radiation (UVB super-hypersensitive) than even the hypersensitive Asian rice Surjamkhi, due to polymorphism of S^{78}-R^{126}-A^{283}-Q^{296} in comparison to P^{78}-Q^{126}-G^{283}-Q^{296} of UVB resistant Asian rice Sasanishiki. The transgenic rice African rice plant generated here, TOG12380-OxPHR, have significant improved in UVB resistance compared with WT plant due to high CPD photolyase activity. On the other hand, UVB pretreatment on S-C line and TOG12380-OxPHR, enhances *M. oryzae* susceptibility, and less activation of PR genes. Thus, the low CPD photolyase activity of Surjamkhi and TOB7307 could be one of the beneficial adaptation strategies for combating the most dangerous stresses in tropical, such as pathogens, as shown here on rice blast fungus *M. oryzae* (Fig. 3F). This study offers knowledge for development of UVB and *M. oryzae* resistant rice, with high yield and productivity.

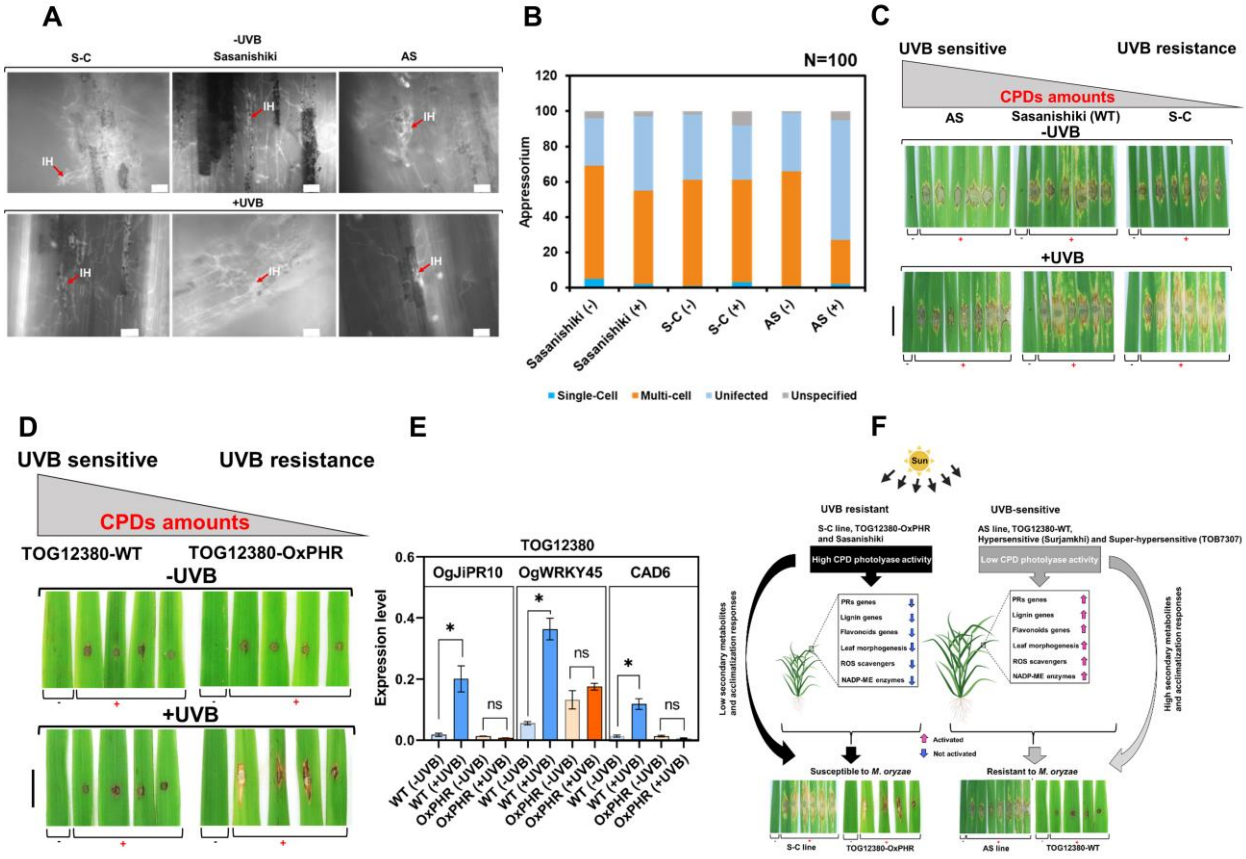

Figure 3. **UVB pretreatment enhances *M. oryzae* susceptibility of CPD photolyase overexpress plants (S-C line and TOG12380-OxPHR)**. (A) Visualization of invasive hyphae structure of Sasanishiki, S-C and AS. Rice leaf sheaths prepared from plants with or without 12 hours UVB radiation pretreatment were stained by aniline blue staining to visualize the structure of invasive hyphae of Ai79-142 after 72 h development within rice leaves. IH, invasive hyphae, scale bars = 20 µm. (B) Number of appressorium-mediated penetration and infectious hyphae development of Ai79-142 in Sasanishiki, S-C and AS. The total number of appressorium is indicated (top right corner, N = 100). Infectious growth was observed at 48 hour post inoculation (hpi) as described above. (C) Blast disease assay of plants pretreated with or without 12 hours of 0.4 W m^{-2} UVB radiation was performed by punch inoculation of *M. oryzae* strain Ai79-142 on 6- to 8- week old leaves from Sasanishiki, S-C and AS lines. (D) Punch inoculation assay of wild type (TOG12380-WT) and transgenic CPD photolyase overexpress African rice (TOG12380-OxPHR). (E) Expression level of OgJiPR10, OgWRKY45 and CAD 6 PR defence genes on TOG12380-WT and TOG12380-OxPHR plants with or without UVB pretreatment. The expression level was performed by qRT-PCR. Values are the mean ± SD. N = 6-8 replicates in B and C; ns, not significant while * and different letters indicate significant differences determined by the Tukey-Kramer test ($P < 0.05$). (F) Summary of relationship between CPD photolyase activity and resistant to rice blast fungus *M. oryzae*.

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論文審査結果の要旨

地球規模での食料問題や環境問題は、アフリカ地域にとって深刻な問題であり、環境変動に左右されない安定的、かつ生産性の高いイネの開発が強く求められている。アフリカで栽培されているイネ品種は、アジアイネ（*Oryza sativa*）とは異なり、アフリカ固有のアフリカ西部地域に自生した *Oryza glaberrima* を起源とする野生イネから栽培化され、アフリカという独特な環境に適応し、様々な環境ストレスに強い形質を示すことが知られている。したがって、生物にとって有害な太陽紫外線 UVB 量が高い地域で栽培されているアフリカ固有のイネ品種は、太陽紫外線 UVB に対しても強い抵抗性を示すと考えれていたが、その詳細は不明であった。そこで、Gideon Sadikiel Mmbando 氏は、アフリカイネの太陽紫外線 UVB による障害と UVB に対する耐性機構を明らかにすることを目的に、アフリカ各地で栽培されているイネ 15 品種の UVB 抵抗性を調査しました。その結果、調査した大部分のアフリカイネ品種は、アジア各地で栽培されているイネ品種よりも高い UVB 感受性を示すことを見出しました。さらにその原因を調べたところ、UVB によって誘発される DNA 損傷（シクロブタン型ピリミジン二量体）を修復する酵素（CPD 光回復酵素：PHR）が、アジアのイネ品種には見られない固有のアミノ酸配列を有しており、この固有なアミノ酸配列が PHR の活性を低下させ、結果として UVB 感受性を導いていることを発見しました。これらの研究成果は、国際誌である Scientific Reports に Mmbando G.S. et al. として受理され、高い評価を受けました。

またさらに、これらの結果を踏まえ、なぜ PHR の活性が低く UVB 感受性の高いイネが UVB 量の高いアフリカや東南アジアで、現在も栽培されているのか？という問いかけに対し、UVB 感受性であることが、これらの地域で栽培するにあたり、有利なことがあるのではないかと考え、他の環境ストレスと PHR 活性との関係について、更なる研究を展開しました。その結果、低い PHR の活性を有するアフリカイネやアジアのイネは、過敏感反応（HR）を活性化し、いもち病菌に対する耐性を獲得できることを PHR 活性を改変したアジア、アフリカイネの組換え体を用いて実証しました。本研究成果は、アフリカにおいて深刻な社会問題となっている穀類の生産性向上に向けた育種、品種開発の新たな方向性を提示することが大いに期待されます。

以上のことから、本研究成果は、自立して研究活動を行うに必要な高度の研究能力と学識を有することを示していると判断し、Gideon Sadikiel Mmbando 氏提出の論文は、博士（生命科学）の博士論文として合格と認める。